

Substrate-specific clades of active marine methylotrophs associated with a phytoplankton bloom in a temperate coastal environment

Diversity of active marine methylotrophs

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ABSTRACT

Marine microorganisms that consume one-carbon (C₁) compounds are poorly described, despite their impact on global climate via an influence on aquatic and atmospheric chemistry.

25 This study investigated marine bacterial communities involved in the metabolism of one-carbon compounds. These communities were of relevance to surface seawater and atmospheric chemistry in the context of a phytoplankton bloom that was dominated by phytoplankton known to produce dimethylsulfoniopropionate. In addition to using 16S rRNA gene fingerprinting and clone libraries to characterize samples taken from a bloom transect in July 2006, seawater

30 samples from the phytoplankton bloom were incubated with ¹³C-labelled methanol, monomethylamine, dimethylamine, methyl bromide, and dimethylsulfide to identify microbial populations involved in turnover of C₁ compounds using DNA stable isotope probing (DNA-SIP). The ¹³C-DNA samples from a single time point were characterized and compared using denaturing gradient gel electrophoresis (DGGE), fingerprint cluster analysis and 16S rRNA gene

35 clone library analysis. Bacterial community DGGE fingerprints from ¹³C-labelled DNA were distinct from those obtained with the DNA of the non-labelled community DNA and suggested some overlap in substrate utilization between active methylotroph populations growing on different C₁ substrates. Active methylotrophs were affiliated with *Methylophaga* spp. and several clades of undescribed *Gammaproteobacteria* that utilized methanol, methylamines (both

40 monomethylamine and dimethylamine) and dimethylsulfide. Ribosomal RNA gene sequences corresponding to populations assimilating ¹³C-labelled methyl bromide and other substrates were associated with *Alphaproteobacteria* (e.g. *Rhodobacteraceae* family), *Cytophaga-Flexibacter-Bacteroidetes*, and unknown taxa. This study expands the known diversity of marine

methylotrophs in surface seawater and provides a comprehensive dataset for focussed cultivation
45 and metagenomic analyses in the future.

INTRODUCTION

Methylotrophic bacteria represent an important functional guild, contributing to the metabolism and assimilation of one-carbon (C_1) compounds. As the carbon sources that they
50 depend on in the marine environment are present at low concentrations, characterizing marine methylotrophs has involved the use of enrichment and cultivation approaches with a variety of C_1 substrates. The C_1 substrates of relevance to the marine environment include methane, methanol, methylated amines, methyl halides and methylated sulfur compounds. Methane is supersaturated in surface seawater and several studies have isolated methanotrophs from the
55 marine environment (14, 16, 27, 46). Methyl halides are produced by a number of phytoplankton species (e.g. 41) and these ozone-depleting compounds have been used to isolate methylotrophic *Alphaproteobacteria* that belonged to the *Roseobacter* clade (42, 44). Methanol represents a marine C_1 substrate derived from phytoplankton (13) and the atmosphere (7), which may be actively metabolised by marine methylotrophs (21). Methanol has been estimated at between 100
60 nM (47) and 300 nM (10) and has been directly measured in one study, ranging between 50-250 nM in several tropical Atlantic samples (53). Enrichment and isolation studies using methanol as a sole carbon source have generated molecular fingerprint phylotypes and characterised isolates of *Methylophaga* spp. (*Gammaproteobacteria*). *Methylophaga* spp. have also been isolated using dimethylsulfide (DMS; 8, 43) and can grow on monomethylamine (e.g. 23), both of which occur
65 at nM concentrations in surface seawater (11, 24). Together, these cultivation-based approaches have revealed the presence of organisms capable of C_1 cycling in the marine environment. Their

involvement in methylotrophic metabolism *in situ* can be experimentally addressed using stable isotope probing (39).

DNA stable-isotope probing recently identified *Methylophaga*-like organisms as active
70 methylotrophs that assimilated methanol and methylamine in surface waters of the English
Channel (35). This study also demonstrated that 16S rRNA gene sequences representing clades
of uncultivated *Gammaproteobacteria* were also retrieved from the heavy DNA for each of these
compounds that clustered close to *Methylophaga*. A SIP experiment with methanol substrate
dilution to concentrations anticipated to reflect those *in situ* (33) confirmed the involvement of
75 *Methylophaga* spp. in methanol consumption and retrieved functional genes involved in
methanol metabolism from these active methylotrophs using metagenomic libraries.

The goal of the current study was to extend our previous observations that were made
during non-bloom conditions, by studying methylotrophic populations in the context of a
phytoplankton bloom dominated by *Emiliana huxleyi* and *Karenia mikimotoi* (formerly
80 *Gyrodinium aureolum*). Both coccolithophores (e.g. *Emiliana*) and small dinoflagellates (e.g.
Karenia) are associated with dimethylsulfoniopropionate (DMSP) production (22, 28) and
phytoplankton blooms are known to produce relevant C₁ compounds or their precursors,
including methanol (13), methylated sulfur compounds (24), and methyl halides (41). As with
our previous marine SIP studies (33, 35), seawater samples were incubated with methanol and
85 methylamine, and in this investigation, SIP incubations were also carried out with ¹³C-labelled
methane, dimethylamine, methyl bromide and DMS in order to identify microbial populations
that are actively involved in the cycling of these C₁ compounds during phytoplankton blooms *in situ*.

MATERIALS AND METHODS

90 *Bloom sampling.* A transect across a phytoplankton bloom dominated by *E. huxleyi* and *K.*
mikimotoi (D. Schroeder, personal communication) was sampled in the English Channel
bordering the south coast of the UK. Surface seawater was taken from inside the bloom (49.3222
N, 5.1446 W to 49.5105 N, 5.1217 W), at the edge of the bloom (49.5472 N, 4.3966 W to
49.5523 N, 4.4045 W), and outside the bloom area (50.1158 N, 4.1998 W to 50.1053 N 4.2062
95 W). The distances between the beginning and end of sampling differed for the three sampling
stations and were 1.3 km, 0.8 km, and 20 km for the outside, edge, and inside of bloom,
respectively. All samples were taken between 10.00 h and 22.00 h on July 26, 2007. Water
samples were returned to the laboratory and aliquots were taken for filtration (for DNA
extraction) and to establish SIP incubations on July 27, 2006. Upon arrival, multiple aliquots of
100 approximately one-litre were filtered through 0.2- μ m Sterivex filters (Durapore, Millipore) and
frozen at -80°C until processed for nucleic acid extraction.

Incubation with ^{13}C labelled substrates. Samples taken from the edge of the bloom were chosen
to set up SIP incubations with several ^{13}C -labelled C_1 substrates. Seawater sample aliquots of
105 750 ml were added to 1-liter serum bottles with the addition of 0.1% (750 μ l) marine ammonium
mineral salts medium (MAMS; modified from 12) and substrate. A total of 75 μ mol of ^{13}C -
labelled substrate was added to bottles for methanol, monomethylamine, dimethylamine, methyl
bromide, and methane (final concentration of 100 μ M assuming complete dissolution). For
dimethylsulfide, 187.5 μ mol of substrate were added to make up a final concentration of
110 250 μ M. All serum bottles were crimp-sealed with butyl rubber bungs to prevent loss of volatile
substrates. All ^{13}C -labelled compounds were 99% or greater purity and obtained from Cambridge
Isotope Laboratories (Hook Hampshire, UK) except methylated amines (Sigma, Gillingham,

UK). Dimethylsulfide ($^{13}\text{C}_2$ -labelled) was prepared by a method adapted from that for labelled dimethylsulfoxide synthesis (5). Sodium sulfide nonahydrate (6.5 g) was dissolved in 6.5 mL of sterile deionised water in a glass test-tube and cooled to 0°C in an ice-water bath with vigorous stirring. Subsequently, 5 g of ^{13}C -methyl iodide (Cambridge Isotope Laboratories LTD, Andover, MA) was added dropwise over a period of 30 min prior to incubating the reaction mixture at 0°C for 5 h with stirring. Five mL of each 2 M sodium hydroxide solution and 1 M sodium thiosulfate solution were added and the reaction vessel was then connected to a receiving tube held at -170°C in liquid nitrogen and the reaction mixture allowed to warm to 40°C in a water bath. The $^{13}\text{C}_2$ -dimethylsulfide was distilled from the reaction mixture for 90 minutes and then re-distilled into a sterile receiving vessel for 1 hour. Sterile deionised water was added to the receiving vessel to dissolve the $^{13}\text{C}_2$ -dimethylsulfide and the resulting solution transferred, with washings, to a sterile 1-l serum vial which was then sealed with a butyl rubber bung. The concentration and purity of the $^{13}\text{C}_2$ -dimethylsulfide solution were assessed by gas chromatography with a flame ionization detector. A total of 250 ml of a 7 mM solution of pure $^{13}\text{C}_2$ -dimethylsulfide was obtained.

For all substrates, parallel incubations were set up as ^{12}C -unlabelled controls and ^{13}C and ^{12}C -substrate incubations were harvested at a single time point. With the exception of monomethylamine and dimethylamine, substrate utilization was monitored by gas chromatography, using a flame ionization detector. Measurement of DMS and methyl bromide concentrations in sterile seawater controls confirmed that the degradation observed in SIP incubations was due to a biological processes and not due to chemical decomposition. The concentrations of the methylamines were assumed to mirror those of methanol; recovery of ^{13}C -DNA from methylamine and dimethylamine incubations confirmed that methylated amines

had been assimilated. Following substrate depletion, SIP incubations were filtered through 0.22- μ m Sterivex filters and frozen at -80°C until processed for nucleic acid extraction.

DNA extraction, SIP gradient centrifugation and fractionation. Total nucleic acids were extracted directly from Sterivex filters according to a previously described protocol (35). Briefly, lysozyme, proteinase K and sodium dodecyl sulfate (SDS) were used to lyse cells and lysates were transferred to 15-ml phase lock tubes (Qiagen, West Sussex, UK) for phenol-chloroform and chloroform extractions. Purified DNA was quantified on a 1% (w/v) agarose gel. Aliquots (1-5 μ g) of DNA extracts from each of the SIP incubations were added to cesium chloride (CsCl) solution (average density of ~ 1.725 g ml⁻¹) and transferred to an ultracentrifuge gradient tube for centrifugation and fractionation as previously described (36). Briefly, tubes were added to a Vti 65.2 rotor (Beckman Coulter, Fullerton, CA) and centrifuged at 44,100 rpm (177,000 g_{av}) for 40 h at 20°C. Gradients were fractionated from bottom (fraction 1; highest density) to top (fraction 12; lowest density) into 425- μ l fractions. DNA was purified from CsCl and quantitatively recovered by precipitation with glycogen (20 μ g) and polyethylene glycol (30% PEG 6000 and 1.6 M NaCl). Purified DNA was suspended in 30 μ l of sterile LoTE buffer (3 mM Tris at pH 8, 0.2 mM EDTA) and 5- μ l aliquots were run on a 1% (w/v) agarose gel for quantification and to identify the distribution of labelled ¹³C-DNA relative to background unlabelled ¹²C-DNA (Figure S1 in the supplemental material). These data indicated that the ¹³C-labelling of DNA was very high for methanol, monomethylamine, and dimethylsulfide incubations; most of the DNA for these ¹³C-incubated samples eluted in heavy fractions (fractions 7 and 8; ~ 1.725 - 1.730 g ml⁻¹). The detection of ¹³C-labelled DNA confirmed that the substrate was incorporated into microbial biomass. For dimethylamine and methyl bromide incubations, the extent of DNA labelling was

less pronounced. For methyl bromide incubations, there was almost no difference between the
smears of DNA across gradients associated with ^{12}C and ^{13}C methyl bromide SIP incubations
(Figure S1 in the supplemental material).

Denaturing gradient gel electrophoresis (DGGE). For each fractionated gradient, two fractions
were selected for analysis of the 'heavy' (^{13}C) DNA (fractions 7 and 8; $\sim 1.725\text{--}1.730\text{ g ml}^{-1}$), and
one fraction was selected for the characterization of 'light' (^{12}C) DNA (either fraction 11 or 12;
 $\sim 1.710\text{--}1.705\text{ g ml}^{-1}$). One- μl aliquots of gradient fractions were used as template for PCR to
obtain 16S rRNA gene fragments suitable for DGGE analysis. Each 50- μl reaction mix consisted
of 25 pmol each of primers 341f-GC and 534r (31), 1 X $(\text{NH}_4)_2\text{SO}_4$ buffer (Fermentas, York,
UK), 1.5 mM MgCl, 33.6 μg non-acetylated bovine serum albumin (Sigma, Gillingham, UK), 40
nmol dNTPs, 1.25 U *Taq* polymerase (Fermentas). The reaction tubes were loaded directly into
the block at 95°C (simplified hot start), followed by an initial denaturation at 95°C for 5 min and
30 cycles of 94°C for 1 min (denature), 55°C for 1 min (anneal), and 72°C for 1 min (extension).
A final extension at 72°C for 7 minutes was followed by a holding step at 10°C . Five- μl aliquots
were quantified on a 1% (w/v) agarose gel.

For DGGE, 5- μl aliquots (100-300 ng) were run on a 10% polyacrylamide gel with a 30-
70% denaturing gradient (100% denaturant is 7.0 M urea and 40% deionized formamide)
according to the D-Code System instructions (Bio-Rad, Hercules, CA, USA). Gels were run
overnight (14 h) at 85 V, then stained for 1 h in SYBR Green I (Invitrogen, Paisley, UK). Gel
images were captured with a FLA-5000 imaging system (Fujifilm, Tokyo, Japan). Bands
selected for sequence analyses were sampled from the gel by means of sterile pipette tips and
amplified from the gel using the PCR conditions described above for DGGE. Sequencing was

done with the 341f primer and the BigDye terminator version 3.1 kit (Applied Biosystems, Foster City, USA) and the sequencing products were run on an ABI PRISM 3130 x l Genetic Analyser (Applied Biosystems) by the Molecular Biology Service, University of Warwick.

185 DGGE band sequences were approximately 150 bases in length.

For determining the relatedness of the DGGE fingerprints, gels were imported into Gelcompar II (Applied Maths, Sint-Martens-Latem, Belgium) and normalized to ladder bands and additional internal standard bands. A UPGMA dendrogram was generated by performing a Pearson correlation on background-subtracted densitometric curves which takes band intensities
190 into account. The output of the clustering analysis was independent of the input order of DGGE fingerprints.

16S rRNA gene libraries. Clone libraries of bacterial 16S rRNA genes were generated from the original seawater samples (outside bloom, edge of bloom, inside of bloom; 36 clones sequenced
195 from each) and for the heavy DNA associated with the five substrates (methanol, monomethylamine, dimethylamine, methyl bromide, dimethylsulfide) that yielded ¹³C-labelled DNA (24 clones sequenced from each). The PCR to amplify the 16S rRNA gene used primers 27f and 1492r (25) and the same amplification reaction as for DGGE except with an extension time of 1.5 min. Products were cloned into the TOPO-TA vector according to the manufacturer's
200 protocol (Invitrogen). Screening was done as described previously (37) and cloned 16S rRNA gene inserts were sequenced at the Edinburgh node of the NERC Molecular Genetics Facility using the 27f primer. The program Pintail (3) was used to identify suspected chimeras and identified one heavy-band sequence which was likely chimeric in origin and several water library sequences that were likely chimeric, these sequences were excluded from further analyses. For

205 seawater samples, classification of 16S rRNA gene sequences was done using the RDP-II classifier (52) after manually verifying base calls. For 16S rRNA gene libraries constructed using ¹³C-DNA from SIP experiments, manually verified 16S rRNA gene sequences were compared to Genbank (6) to retrieve three closest matches for each library sequence. Sequences were aligned within Arb (29) and an alignment was exported to MEGA4 (48). Evolutionary distances were
210 computed using the Maximum Composite Likelihood method (49) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 466 nucleotide positions in the final dataset. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (9). The
215 tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. In the absence of cultivated methylophilic organisms that fell within the groups of 16S rRNA genes derived from SIP experiments, clades were defined based on the consistent association with particular substrates but a specific cut-off value was not used.

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Genbank sequence deposition. All sequences were deposited in Genbank for the marine samples taken from the edge (EU399242-EU399272), inside (EU399273-EU399306) and outside (EU399307-EU399340) of the phytoplankton bloom. The 16S rRNA gene clone library sequences from heavy DNA were deposited with the following accession numbers for SIP
225 incubations with dimethylamine (EU399341-EU399364), dimethylsulfide (EU399365-EU399386), methyl bromide (EU399387-EU399407), monomethylamine (EU399408-EU399428) and methanol (EU399429-EU399451). DGGE band sequences from heavy DNA

were deposited with the following accession numbers for SIP incubations with methanol (EU399452-EU399457), monomethylamine (EU399458-EU399464), dimethylamine (EU399465-EU399469), dimethylsulfide (EU399470-EU399474) and methyl bromide (EU399475-EU399477).

RESULTS

Phytoplankton bloom microbial community analysis. This study was conducted on samples from an extensive mixed phytoplankton bloom with a predominance of both *Emiliania huxleyi* and *Karenia mikimotoi* (D. Schroeder, personal communication). Based on remotely sensed observations from the day prior to sampling, three sampling stations within the western English Channel were selected to represent areas of varying chlorophyll concentrations (Figure 1A), indicating regions internal to the bloom ('inside'), on the edge of the bloom ('edge') and external to the bloom ('outside'). Prior to assessing the methylotrophs in the bloom (edge sample), we assessed the background bacterial community composition of the three water samples using 16S rRNA gene fingerprinting (Figure 1B) and clone libraries (Figure 1C). The DGGE profiles indicate that the bacterial communities of these three water samples were represented by unique predominant band phylotypes, although several bands were shared between the three samples (Figure 1B). Almost all sequences collected from the 16S rRNA gene clone libraries were most similar to Genbank sequences derived from other marine surface water samples, reflecting a composition similar to previous studies (data not shown). All libraries were dominated by *Alphaproteobacteria* and *Cyanobacteria*, although *Bacteroidetes* were also prevalent in the 'Inside' and 'Edge' libraries (Figure 1C). Overall, the communities shared similar division-level

250 composition but also indicated that local sample heterogeneity existed across this relatively short bloom transect.

DNA-SIP incubations. Enrichment incubations with six C₁ substrates were established on the day following sampling (day 0) with substrate concentrations of 100 µM (250 µM for DMS).

255 Substrate had been depleted by day 3 in methanol incubations and these were filtered for DNA extraction as were those containing methylamines. Approximately 110 µM of ¹²C and ¹³C-DMS were consumed by the fourth day (data not shown) and these incubations were subsequently sacrificed for DNA extraction. Methyl bromide incubations (¹²C and ¹³C) had consumed >90% of the 100 µM of substrate originally present by day 18 and were filtered for DNA extraction.

260 Changes in headspace concentrations of methane (100 µM total in bottle; ~0.63% in headspace) for seawater incubations with methane were unchanged for several months (data not shown) and these incubations were not analyzed further.

16S rRNA gene fingerprinting of DNA from SIP experiments. Denaturing gradient gel

265 electrophoresis (DGGE) was used to profile the bacterial communities associated with ‘heavy’ and ‘light’ fractions for both ¹²C-control samples and ¹³C-incubated samples. The ¹²C incubated samples showed no profile difference between the ‘heavy’ and ‘light’ fractions (data not shown), whereas unique fingerprints were evident for all C₁-substrate incubations (Figure 2). As expected, the fingerprints for the light fractions of all tubes clustered together. The ‘background’
270 bacterial communities in each SIP incubation were more similar to one another than to the ‘heavy’ ¹³C-fraction fingerprints of the same incubation. However, for the methyl bromide SIP incubation, the fingerprint of the ‘heavy’ DNA was less clearly unique from the light DNA than

for the other substrate incubations, reflecting that only a small amount of DNA was labelled and was just detectable above background ^{12}C -DNA. All other DGGE fingerprints from heavy
275 fractions (fractions 7 and 8) of ^{13}C -substrate incubations clustered in distinct clades apart from the 'light' DNA, with monomethylamine and methanol fingerprints clustering closely, with some similarity to the dimethylamine fingerprints. Dimethylsulfide ^{13}C -DNA fingerprints were distinct from all other patterns in this study, reflecting a unique composition of active methylotrophs enriched in these SIP incubations. Individual bands from fingerprints representing 'heavy' DNA
280 from $^{13}\text{C}_1$ incubations were selected for PCR reamplification and sequencing. These sequences were used to assign band sequences to specific SIP-related 16S rRNA gene clades derived from this study and from a previous SIP study that was carried out during non-bloom conditions in the English Channel (35). The results indicate that *Methylophaga* spp. were associated with methyl bromide, methanol and methylamine SIP incubations, whereas additional clades were affiliated
285 with dimethylamine and dimethylsulfide, likely contributing to their more distinct fingerprint profiles (Figure 2).

16S rRNA gene clone libraries of ^{13}C -DNA. As the diversity of active methylotrophs was anticipated to be relatively low, 24 clones were sequenced from each library associated with SIP
290 incubations with each of the five substrates analyzed in this study. The results of the sequencing confirmed relative low diversity of methylotrophs within each SIP incubation, but across the different substrates applied indicated a broad diversity of active marine methylotrophs in this study.

295 *Methanol-assimilating phylotypes*. Phylogenetic analysis demonstrated that sequences associated with SIP incubations with methanol clustered in the *Methylophaga* spp. clade of the *Gammaproteobacteria* together with sequences from a previous SIP incubation (35) and with several characterised *Methylophaga* isolates (Figure 3, Figure S2 in the supplemental material). In addition, one cloned 16S rRNA gene sequence obtained from the methanol SIP was a member
300 of a clade of unknown phylogenetic affiliation, which also contained one DMS and DMA-SIP derived cloned 16S rRNA gene respectively. The methanol clade identified in a previous methanol SIP experiment (Figure 3; Figure S2 in the supplemental material) has closest affiliation to *Gammaproteobacteria* sequences in Genbank that were retrieved from multiple marine Arctic surface sediments or detected on the surface of submerged artificial substrates
305 incubated in marine water near China. This clade however, was not detected in the current study.

Phylotypes assimilating methylated amines. As with the methanol SIP incubation, a previously characterised clade of sequences associated with a monomethylamine SIP incubation (35) was also represented by sequences from the monomethylamine SIP from the current study, and also
310 from the dimethylamine SIP incubation. In particular, 22 of the 24 sequences generated from the dimethylamine SIP incubation and most of the corresponding DGGE band sequences (Figure 2) fell within this clade (Figure 3, Figure S3 in the supplemental material). This clade also contained several sequences isolated from Arctic sediment (Li et al, unpublished), a mangrove ecosystem (Liao et al. 2007, Microb Ecol. 54(3):497-507), and a deep-sea coral (Penn et al
315 unpublished) and a strain isolated from the Yellow Sea (Kim and Cho, unpublished, Genbank accession EF468718). Additional 16S rRNA gene sequences obtained from the

monomethylamine SIP incubation belonged to the *Methylophaga* clade many species of which can grow on methylated amines.

320 *Phylotypes assimilating DMS*. Almost all 16S rRNA gene sequences derived from the dimethylsulfide SIP ‘heavy’ DNA were nearly identical and formed an additional clade with low relative diversity (Figure 3; Figure S4 in the supplemental material). The DMS clade was most closely related to the methanol SIP clade associated with the *Gammaproteobacteria* and identified in a previous study (35) and shared close similarity (96%) with sequences retrieved
325 from clone libraries associated with DMS-enriched seawater samples from the Sargasso Sea (51) and was approximately 91% similar to the *Methylophaga* sp. clade, based on the percent similarity between sequences DMS_584_22 and *Methylophaga marina* (accession number X95459) over 722 bases. Another sequence from the ‘heavy’ DNA of the DMS SIP was affiliated with a clade of unknown phylogeny.

DISCUSSION

The study site was chosen based on the mixed *Emiliania* and *Karenia* bloom that occurred in the English Channel in July of 2006. The growth of phytoplankton in oceanic surface
335 water has been associated with the direct or indirect production of methanol (13), methylamines (reviewed in 32), methyl halides (2, 4, 30, 41), methylated sulfur compounds (19, 20, 26) and methane, through decomposition (15, 38). In sampling from the edge of the bloom for SIP analysis (Figure 1), the objective was to retrieve sequences of methylotrophs relevant to bloom C₁ substrate production. Although the sample chosen was relevant to C₁ metabolism, it is

important to note that the substrate concentrations (100 μM) were far higher than those normally present in marine surface water samples. This was done because for a previous bloom in Bergen, Norway, the application of C_1 substrates at low μM concentrations did not result in the detection of ^{13}C -labelled DNA, possibly due to relatively high bacterial biomass associated with the bloom (Murrell *et al.*, unpublished). In this study, the objective was to identify phylotypes associated with the use of labelled C_1 substrates and the use of elevated substrate concentrations may have biased the results obtained. Typically, SIP experiments require substrate concentrations that exceed those found naturally and the data may have to be interpreted with caution (34). Nonetheless, a comparison of near *in situ* substrate concentrations (1 μM) with a marine methanol SIP incubation detected the same *Methylophaga* spp. phylotypes as detected in the present study (33). As a result, for C_1 substrates in the marine environment, the results may be consistent despite the range of substrate concentrations used. In all SIP incubations thus far, the incubation times were extended to days and an addition of nutrients may have also selected for a fast growing species of methylotrophs. However, the uncultivated methylotrophs detected here are consistently present, which suggests that they do play an active role in C_1 metabolism in coastal marine environments.

This study represents a comprehensive survey of active methylotrophs in a marine surface water sample during a bloom of phytoplankton associated with production of DMSP. The methylotrophs detected in this survey are consistent with the results of our pilot study with only methanol and monomethylamine under non-bloom conditions obtained a year prior to the current sampling event (35); however, use of a wider range of C_1 substrates allowed the identification of a larger diversity of methylotrophs than found previously, including populations assimilating dimethylamine, DMS and methyl bromide. DMS SIP clones obtained were most

365 closely related to clones obtained from DMS enrichments from Pensacola and the Sargasso Sea
by Vila-Costa and colleagues (51), suggesting that the latter had similar metabolic activities and
indeed represented DMS degrading populations. Those sequences were classified as
“uncultivated *Methylophaga*”; however, given the relatively low similarity of the 16S rRNA
gene sequences of these cloned 16S rRNA gene sequences to those of *Methylophaga* isolates
(around 92%) and their distinct clustering supported by bootstrap analysis (see Fig. S2 and S4), it
is also possible that these represent DMS-degrading populations belonging to a different genus.
370 Conversely, none of the DMS SIP clones were closely related to previously isolated DMS-
degrading *Methylophaga* isolates (43), which belonged to the *Methylophaga* clade detected on
methanol, monomethylamine and methyl bromide, strongly suggesting that populations closely
related to the isolated strains may have a preference for other C₁ substrates and/or are
outcompeted by those represented by the DMS clade under the specific incubation conditions.
375 The methyl bromide SIP sequences suggest that methyl bromide may be used by members of the
Methylophaga genus and an organism with a 16S rRNA gene sequence most similar to
Phaeobacter gallaeciensis (formerly *Roseobacter*) within the *Rhodobacteraceae*. The notion that
the *Phaeobacter*-related population degraded methyl halides would be supported by previous
cultivation based identification of marine methyl halide degrading organisms which were closely
380 related (42, 44, 45); however, screening of several *Methylophaga* isolates has failed to show their
ability to degrade methyl halides (Schäfer, unpublished). The observation of *Methylophaga*-like
sequences in the ¹³C-methyl bromide incubation could therefore be due to the slow hydrolytic
conversion of methyl bromide to methanol (1) and subsequent utilisation of the resulting
methanol by these organisms. If *Methylophaga* populations in the methyl bromide incubations
385 became labelled with ¹³C due to uptake of methanol produced by conversion of methyl halides to

methanol, this would further underline their ability to take up methanol at ambient concentrations and support using SIP incubations with elevated substrate concentrations to investigate substrate responsive populations in seawater. Together, these data suggest that marine waters harbour a diverse suite of active methylotrophs that, apart from *Methylophaga* spp. have been unnoticed by previous cultivation studies (8, 17, 23, 42-44) and are almost completely without representation in marine clone libraries. The sequences represented here represent important targets for directed cultivation and focussed activity-based studies of marine methylotrophy.

Given the focus of past marine metagenomic studies on abundant community members, it is perhaps not surprising that few genes (phylogenetic or 'functional') have reflected the predominance of methylotrophic bacteria. Although formaldehyde oxidation genes were identified in the Sargasso Sea metagenomic libraries (50), genes for methane, methylamine, and methanol oxidation were not detected (18). Furthermore, the only presumed methylotroph 16S rRNA gene sequences identified in a marine metagenomic library was from *Methylophilus* spp. and these sequences occurred at ~0.4% of the total 16S rRNA gene dataset from the global ocean survey (40). The contribution of *Methylophilus* to marine C₁ cycling remains unclear and *Methylophilus* spp. have not been detected in ¹³C DNA from the incubations carried out in this study. One possibility is that *Methylophilus* spp. represent *K*-selected organisms that are adapted to concentrations of carbon and nutrients that are lower than those used in this study. Cultivation-based approaches (17), enrichment cultures (43, 51) and SIP incubations (current study; 33, 35) have all demonstrated that *Methylophaga* spp. and related *Gammaproteobacteria* from multiple disparate marine samples (including estuary sediment; unpublished data) are present in the seawater samples and rapidly respond to the presence of C₁ substrates. It is possible that these organisms may represent low-abundance and *r*-selected bacteria that are capable of opportunistic

growth in the presence of relatively high concentrations of growth substrates during
410 phytoplankton blooms, for example.

This study represents a comprehensive cultivation-independent survey of active marine
methylophs and demonstrates that previously unrecognized bacterial groups are present in
seawater, which are capable of responding to the presence of added C₁ substrates. The presence
of numerous clades of presumed substrate-specific methylophs presents a challenge to
415 microbiologists to focus cultivation and quantitative molecular approaches to better understand
the metabolism and distribution dynamics of these organisms with potentially enormous
biogeochemical significance.

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Figure 1. Physical location and bacterial composition of samples. A chlorophyll-*a* satellite

image (A) from July 24, 2006 shows regions of high chlorophyll associated with a mixed bloom dominated by *Emiliana huxleyi* and *Karenia*. (B) Bacterial DGGE fingerprints of samples taken from the locations indicated in (A). (C) Frequency of 16S rRNA gene clones belonging to major phylogenetic groups across the different gene libraries analysed.

Figure 2. DGGE fingerprint comparison of ‘light’ and ‘heavy’ DNA associated with DNA SIP

incubations with different carbon sources (MMA, monomethylamine; DMA, dimethylamine; MOH, methanol; MBr, methyl bromide; DMS, dimethylsulfide). The dendrogram scale bar refers to percent similarity of Pearson correlations between fingerprint densitometric curves.

Shading of the triangle pointers indicates the phylogenetic affiliations of sequenced bands, most of which were associated with clades in Figure 3. Numbers to the bottom right of fingerprints

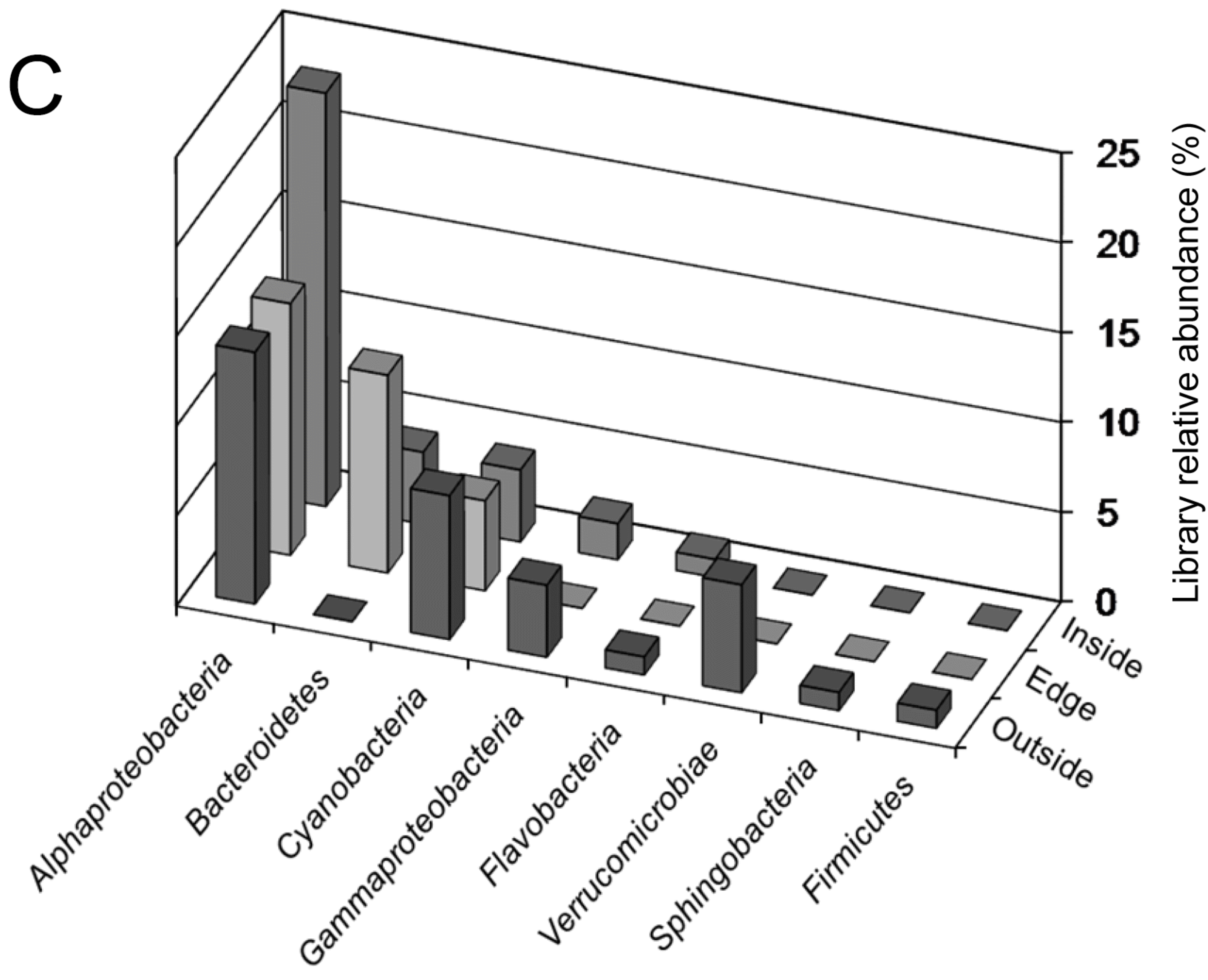
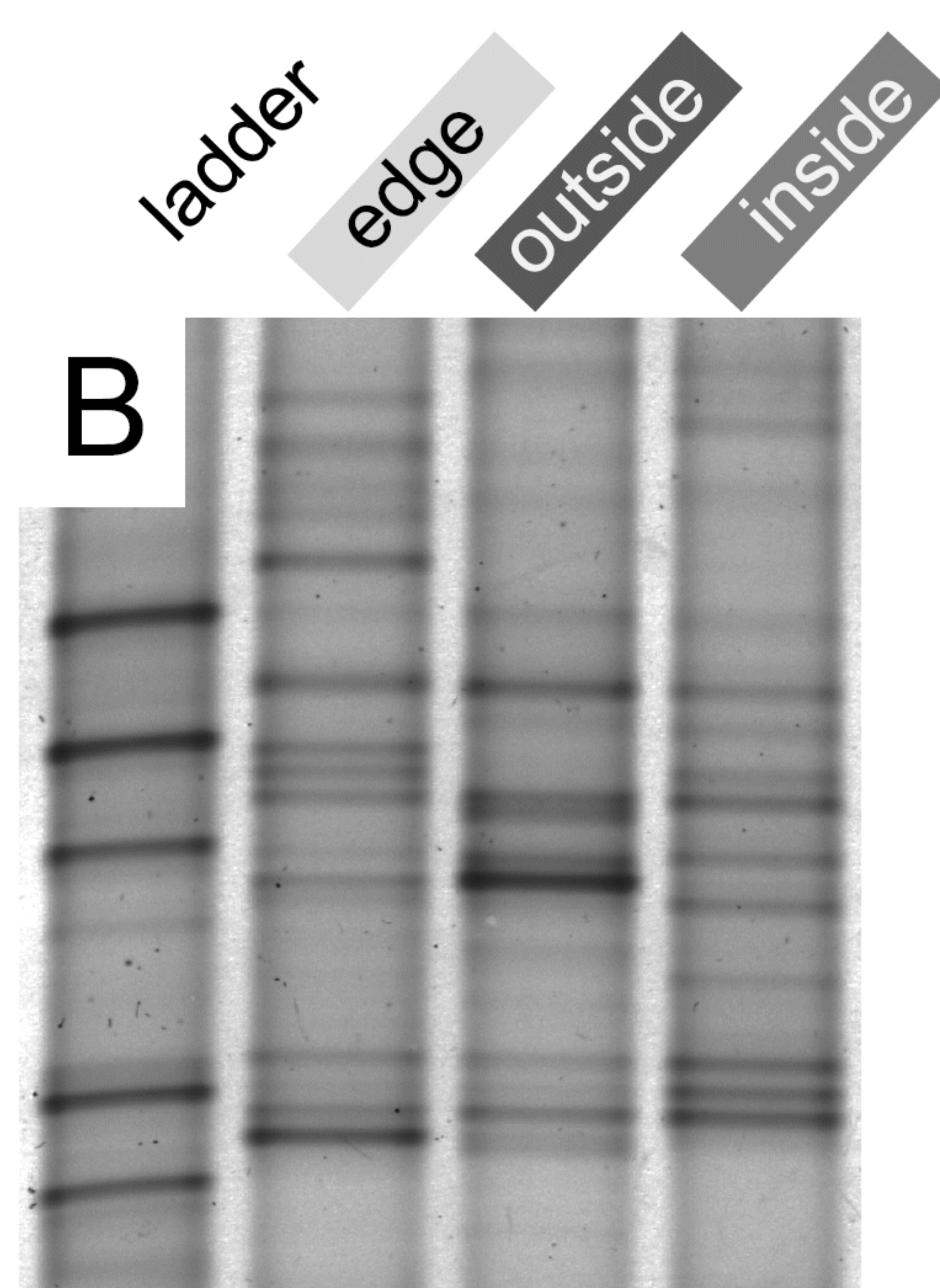
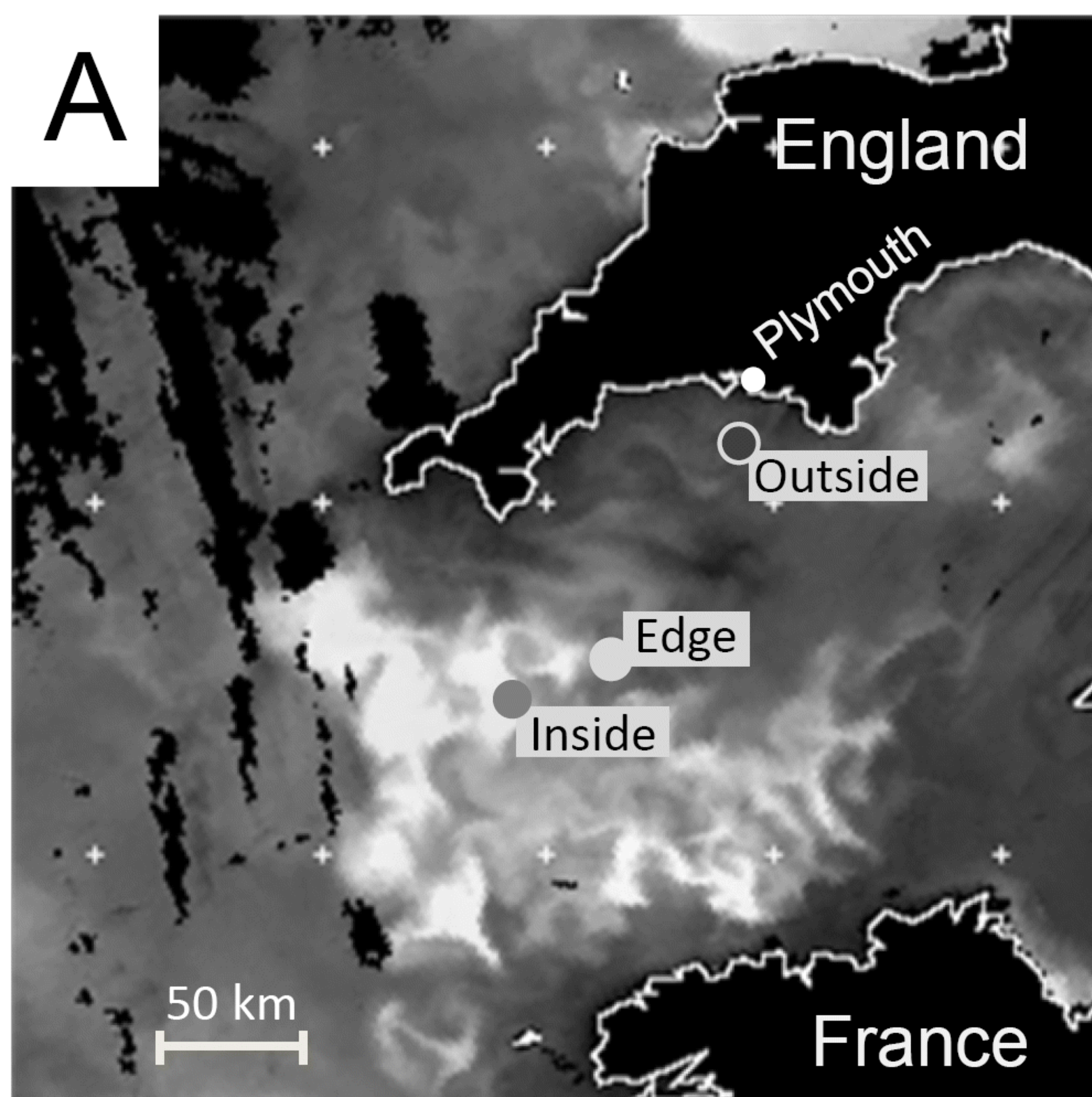
correspond to sequenced bands submitted to Genbank. For example, the open triangle for fraction 7 of the ¹³C-methylbromide SIP (MBr_7) will be labelled MBr_7_3 for the Genbank submission. Several bands were not associated with clades but were affiliated with sequences in Figure 3: MBr_7_3 is identical to MBr_587_7, DMA_7_6 is closest to MBr_587_24, DMS_7_2 is identical to DMS_584_3.

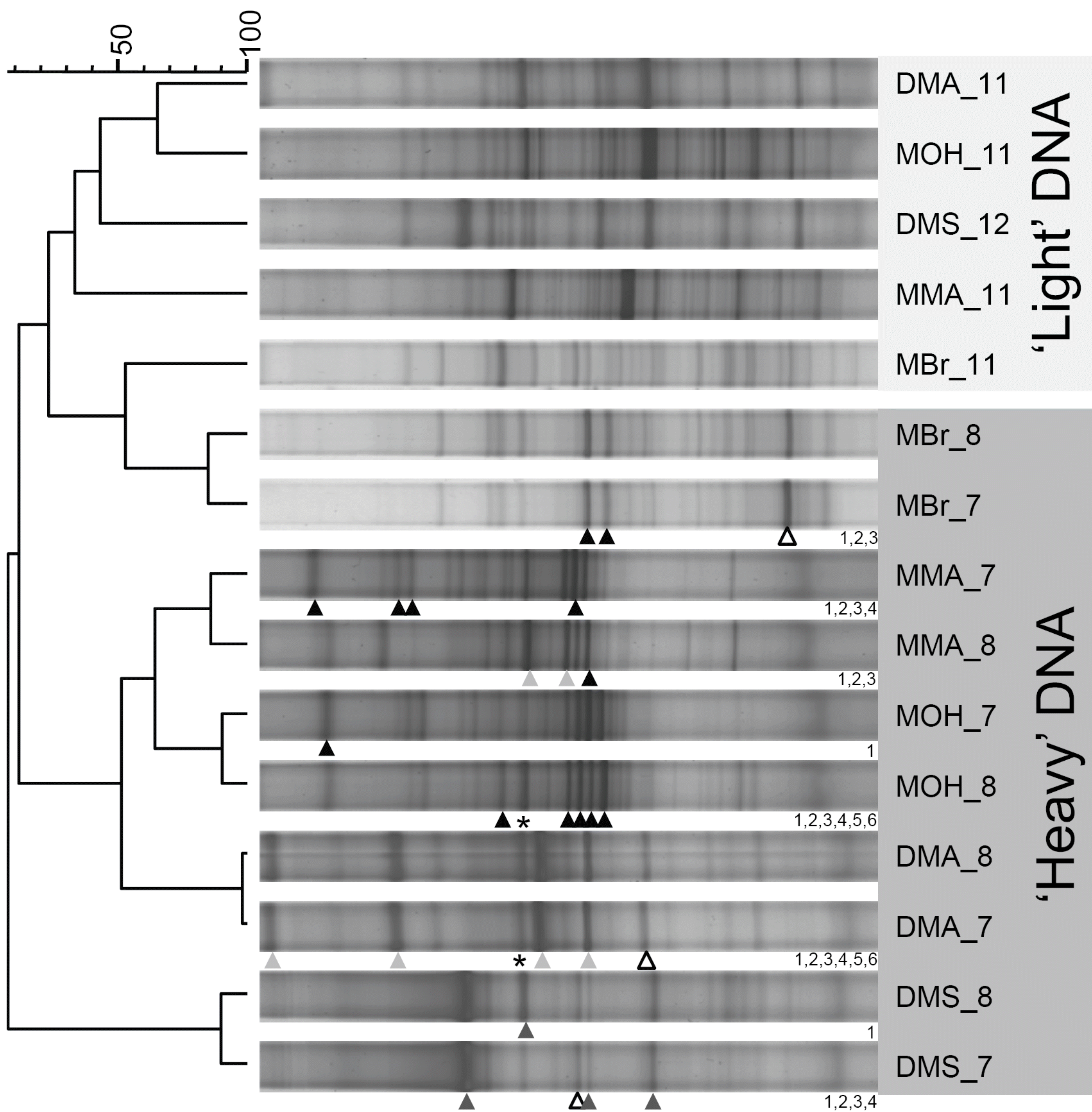
Figure 3. Phylogenetic affiliations of 16S rRNA gene sequences obtained by ¹³C₁-SIP

incubations with methanol (MOH), monomethylamine (MMA), dimethylamine (DMA), methyl bromide (MBr) and dimethylsulfide (DMS) SIP incubations. Selected Genbank sequences from uncultivated clones and reference strains are included for comparison. Bootstrap values are included for all branch points on this neighbour-joining tree. Genbank accession numbers are

610 included within parentheses. The scale bar (within tree) represents 5% sequence divergence. The
collapsed clades are expanded in Figures S2, S3 and S4 found in the supplemental material. The
division-level affiliation of sequences indicated in the boxes along the right-hand side. CFB is
Cytophaga–Flavobacterium–Bacteroides.

615





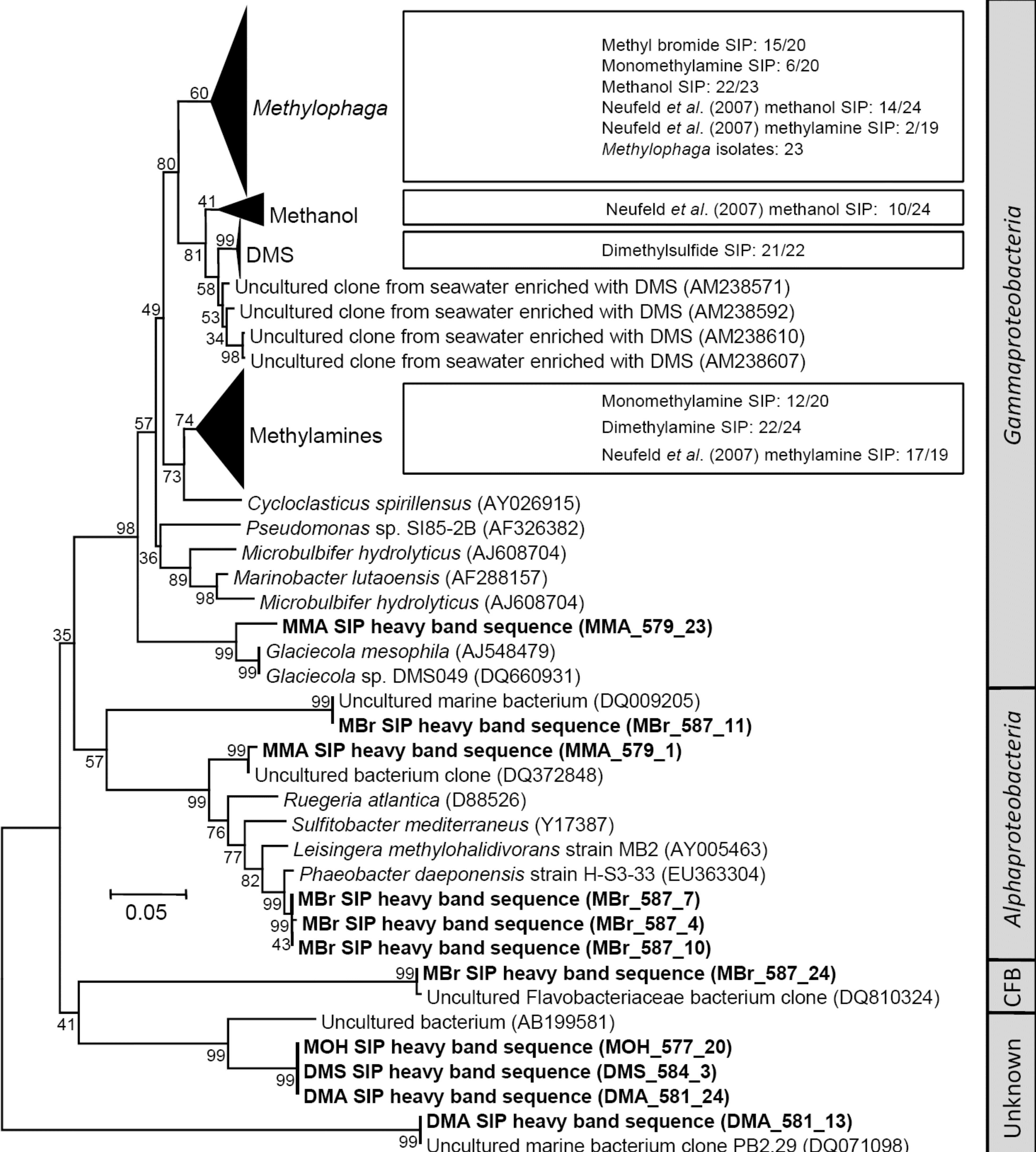
* Failed reaction

▲ *Methylophaga* spp. cluster

▲ Methylamines cluster

▲ Dimethylsulfide cluster

△ Other clades



Methyl bromide SIP: 15/20
Monomethylamine SIP: 6/20
Methanol SIP: 22/23
Neufeld *et al.* (2007) methanol SIP: 14/24
Neufeld *et al.* (2007) methylamine SIP: 2/19
Methylophaga isolates: 23

Neufeld *et al.* (2007) methanol SIP: 10/24

Dimethylsulfide SIP: 21/22

Monomethylamine SIP: 12/20
Dimethylamine SIP: 22/24
Neufeld *et al.* (2007) methylamine SIP: 17/19

Gammaproteobacteria

Alphaproteobacteria

CFB

Unknown